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Research Article

Biocompatibility of Porous Tantalum Material Co-Cultured Bone Marrow Stromal Stem Cells

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Abstract

Objective: The research of observation of canine bone marrow stromal stem cells attached to the surface of the porous tantalum material implanted in dogs and proliferation and *in vivo* biocompatibility.

Methods: Extraction of primary canine bone marrow stromal cells, flow cytometry cell surface CD90, CD44 and CD34 protein, identification of cell types. *In vitro* tests, using the MTT assay the toxicity of porous tantalum canine bone marrow stromal stem cells and observed conditions of stem cells adhered to the porous tantalum material and growth by scanning electron microscopy; *in vivo* experiments, the animal models of bone defects were made by implantation of porous tantalum material, in 6 weeks, 12 weeks animals were sacrificed respectively and the observation of the growth of tantalum metal material with the surrounding tissue was achieved by hard tissue sections stained.

Result: We observed *in vitro* tests, there were no cells adhere to the surface of tantalum material till canine bone marrow stromal cells and porous tantalum material joint training 5d. Joint training 10d, cells dispersed in the surface of a porous tantalum material, there were no connections between cells. Joint training 15d, cells connected into pieces, we could observe that cells secreted large amounts of collagen fibers. *In vivo* tests, a large number of bone marrow stromal cells surrounding porous tantalum material till 6 weeks, there are gaps in the porous tantalum material cells and fibrous tissue of the bone till 12 weeks.

Conclusion: The cells we extracted are determined to the cells of bone marrow stromal cells; the MTT assay proved that porous tantalum was clearly no cytotoxicity. Electron microscopy confirmed that cells and porous tantalum material attached very closely. Vivo tests proved that porous tantalum material has good histocompatibility and bridging role in promoting growth, tantalum material has a role in promoting for repairing defects in the growth of the femoral head. Porous tantalum metal and bone marrow stromal cells has good biocompatibility *in vivo* or *in vitro*.

Keywords: Biological Compatibility; Bone Defect; Marrow Stromal Stem Cells; Porous Tantalum Material

Introduction

Bone defects bring great challenges to the doctors in clinical treatment. Among methods of treating bone defects, such as application of conventional methods like bone grafting (including autografting and allografting), topical application of novel biomaterials and bone tissue engineering, comparatively good one is bone grafting. However, autografting faces limited bone supply, as well as consequences like donor site infection. Allografting is not absolutely safe as well, which may result in infectious diseases [1]. During grafting, patients suffer greatly, thus posing many clinical inconveniences. Bone defects can be cured well if treated timely. Research of biomaterials in recent years has brought new vitality to the treatment of bone defects. Tantalum, as a foreign implant material, possesses prominent biocompatibility, which can provide sufficient and highly stable support if fixed in the bone defect area. This approach can greatly reduce the stress on bone tissues to prevent the femoral head collapse, thereby providing favorable conditions for bone tissue repair [2]. In this paper, *in vitro* and *in vivo* animal studies were conducted using an independently developed porous tantalum material, in order to provide the theoretical support for clinical treatment of avascular necrosis of femoral head.

In this experiment, canine femoral shaft marrow fluid was extracted to obtain Bone Marrow Stromal Stem Cells (BMSCs) by whole marrow centrifugal stratification, which were passaged *in vitro*. The third-generation cells were then collected and cultured jointly with the independently developed porous tantalum material. MTT assay was employed to identify the cytotoxicity of the material, while SEM was used for observing the material, as well

as the adhesion, proliferation and growth of cells. In the *in vivo* experiment, independently developed porous tantalum material was implanted into dogs, which was removed at two-time points to prepare pathological sections. Ingrowth of bone tissues and the porous tantalum material were observed, as well as whether the material had a good histocompatibility in animal body. This experiment provides a theoretical basis for the clinical application of the independently developed porous tantalum material.

Materials and Methods

Experimental Animals

Young healthy male mongrel dogs (animal license No.: SCXK (Liao) 2013-0003), about three months of age, weighing 8.5-10 kg, were provided by the Animal Laboratory of the Dalian Medical University. Prior to experiments, the dogs were adaptively fed for one week in separate rooms with standard diet and watered ad libitum. In this study, experimental animals were processed in accordance with the requirements of "Guidance for the Care and Use of Laboratory Animals" issued by the Ministry of Science and Technology in 2006 [3].

Experimental Instruments and Reagents

Instruments and reagents	Sources
DMEM/F12 medium	Hyclone, USA
FBS	Hyclone, USA
MTT reagent	Amresco, USA
CD90 polyclonal antibody	Biolegend, USA
CD44 polyclonal antibody	Biolegend, USA
CD34 polyclonal antibody	Biolegend, USA
Refrigerated centrifuge	Sigma, USA
Inverted microscope	Olympus, Japan
Cell incubator	Thermo Forma, USA
Flow cytometer	Becton, Dickinson and Company, USA
Scanning electron microscope	Philips, Netherlands

Methods

Animal Grouping and Model Preparation

After purchase, the dogs were adaptively fed for two weeks in our hospital's breeding center. The primary BMSCs were extracted first time and identified with a flow cytometer. In the *in vitro* experiment, cytotoxicity of the independently developed porous tantalum material against canine BMSCs was detected by MTT assay. BMSC adhesion and growth were observed by SEM. In the *in vivo* experiment, the independently developed porous tantalum material was implanted into the animal model of bone defects. Then, 6 and 12 weeks later, respectively, the animals were killed, and growth of the porous tantalum material in the body tissues was observed with inverted microscope using HE-stained hard tissue sections.

In Vitro Cell Culture Observation

Separation and culturing of canine BMSCs: Infantile experimental dogs were anesthetized by intramuscular injection of 0.08-0.1ml xylazine hydrochloride per kilogram, then intercondylar fossa at the inferior femoral end was separated under sterile conditions. 8 mL of bone marrow blood was extracted, anticoagulated with heparin and trimmed into an equal proportion of 16 ml with PBS. Canine BMSCs were separated by density gradient centrifugation, added with 5 mL of DMEM medium, seeded in a 25 mL Petri dish and cultured statically in a 37℃, 5% CO_2 incubator with saturated humidity. At 24 h, medium was replaced at half volume, and at 48 h, the medium was replaced at full volume. When BMSCs adhered to the side wall of petri dish and grew peripherally, medium was changed at full volume. Cells other than adherent cells (red blood cells, suspended bone marrow hematopoietic stem cells and some non-adherent bone marrow stem cells) were removed. Afterwards, medium was changed every 2-3 d. Culturing, amplification and passage of canine BMSCs: When BMSCs were initially cultured for 8-12 days, about 80-90% of cells were adherent and fused. Culture medium was completely replaced and gently dispersed twice with PBS. Petri dish was added with 1.5 ml of 0.25% trypsin, placed in a cell incubator and trypsinized for 4-5 min, followed by observation of cell detachment with an inverted microscope. Cell detachment could also be promoted by patting both sides of culture flask. When 50% of cell turned around and cytoplasmic retraction was seen under microscope, trypsinization was terminated with 4.5 ml of DMEM, and cells were passaged at a ratio of 1: 2 or 1:3. Cell growth was observed daily with an inverted microscope until the adherent cells covered the flask bottom and fused together. In this way, passaged cells were retained repeatedly for later use.

Flow Cytometry Method

Identification of canine BMSCs: The second-generation cells were added with 1.5 ml of 0.25% trypsin, cultured in an incubator for 4 min, gently dispersed uniformly with double medium and centrifuged at 1,000 r/m for 5 min. After discarding the supernatant, cells were concentration adjusted, placed separately into Petri dishes, reacted for 20-30 min at room temperature with CD90, CD44 and CD34 rabbit polyclonal antibodies, then reacted in the dark for 30 min with labeled anti-rabbit IgG secondary antibody. After washing with PBS, the reacted cells were loaded into a flow cytometer for cell identification.

MTT Cell Proliferation Assay

Tantalum rod material was prepared into appropriately sized pieces and placed into a 96-well plate. Cells were seeded into 10 wells of the 96-well plate at a density of 1×10^4 /mL and cultured in an incubator for about 4 d. After placing 0.002 g of MTT into an EP tube, the tube was added with 400 µl of PBS, shaken evenly on a shaker and centrifuged once on a centrifuge. Afterwards, the 96-well plate was removed from the incubator, 10 wells of which were added with prepared MTT at 20 µl per well and cultured in an

incubator for 4 h. 4 h later, MTT in wells was completely replaced. After removing the material, 200 µl of DMSO was added into each well, and the plate was shaken uniformly on a shaker for 10 min. Optical density was measured at 490 nm using an ELISA reader to obtain the optical density and cell proliferation properties. SEM observation of the proliferation and growth of canine BMSCs combined with independently developed porous tantalum

The independently developed porous tantalum was cut into 0.2 cm thick, 0.4cm wide blocks, washed with triple-distilled water, soaked in a sterile cup, dried, autoclaved and then soaked in the culture medium for 24 h for subsequent experimental use. Third-generation cells were seeded into a 24-well plate at a 1.5×10^6/ml density in a suspended state. Then, each well was added with a block of independently developed porous tantalum material, which was spread on the bottom of the plate to enhance the tightness between cells and tantalum material. Next, 1 ml of medium was added, and the plate was co-cultured in a 37℃, 5% CO_2 incubator with saturated humidity. On the 5th, 10th and 15th d of co-culturing, the tantalum blocks were removed (n = 4 in each group), washed in PBS, fixed in 2% glutaraldehyde, dehydrated with gradient ethanol, dried, fixed by surface vacuum metal spraying and placed under a scanning electron microscope for observation of material surface structure and cell adhesion.

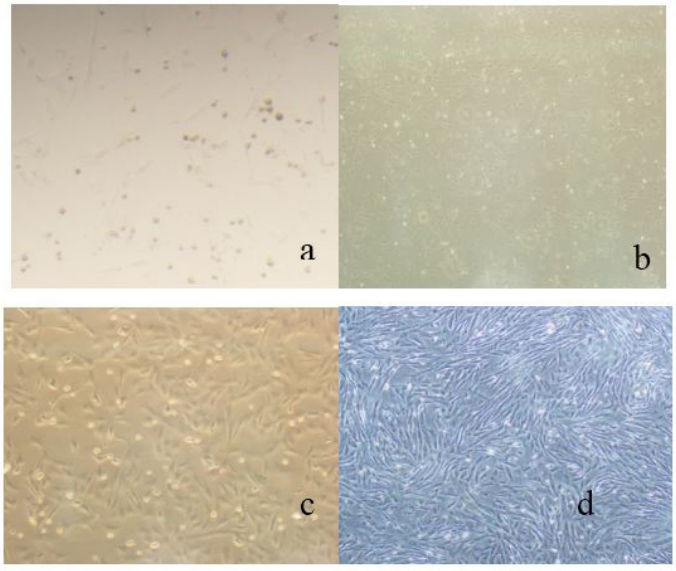
Treatment of Bone Defects with Foreign Porous Tantalum Implant

In the *in vivo* experiment, two-time periods were designed. Two dogs in the experimental group were each implanted with porous tantalum material at the left femoral head, and 6 weeks later, at the right femoral head. 12 weeks later, dogs in two groups were killed, and porous tantalum implantation was observed at two different time points. Canine femoral heads were removed 12 weeks later, fixed in 10% formalin and decalcified in a solution made of formaldehyde, triple-distilled water and nitric acid. About 10 days later after softening of the femoral heads, specimens were removed and cut into 2 mm thick tissue sections with a microtome, followed by observation under an inverted microscope.

Results

Cell Morphology

In the initial culture stage of primary BMSCs, floating cell suspension and erythrocyte debris were observable. Medium was replaced completely 48 h later. 72 h later, medium was replaced, and floating cells were removed. Under inverted microscope, adherent cells increased significantly to form multiple cell colonies of different sizes. Cell morphology varied, presenting fusiform, short fusiform, circular, oval or polygonal shapes; meanwhile, individual cells began stretching (Figure 1a). At 72 h, most adherent cells became long fusiform, with only a few becoming circular or oval; besides, cells exhibited colony growth (Figure 1b). Adherent cells grew rather fast. Latency phase lasted about 3 days, during which cell growth was relatively stable without obvious morphological changes. During logarithmic phase (5-7 days), cell growth was more active than the latency phase, and cells were arranged in a certain direction (Figure 1c). Approximately on 8-10 days, cells entered the proliferation phase. In this phase, cell fusion was close to 90%, spiral changes were seen in morphology, and cells were nearly confluent and tightly attached to each other (Figure 1d).



Figures 1(a-d): (a) Cells observed under microscope (× 200), an anchorage-dependent cells can be seen increase significantly under the microscope after 2d, cell morphology were various , such as fusiform, short spindle-shaped, round, oval or polygonal shape individual form begun to stretch, **(b)** Anchorage-dependent cells presented long spindle-shaped when the 4d, a few were round or oval, **(c)** Anchorage-dependent cells grown quickly, with relatively active cell growth, and arranged to a certain direction form between cells, **(d)** Approximately 8-10d later, cells fused nearly above 90%, spiral growth could be seen , at this time the cells attached to each other closely .

Passaged cells grew faster, with a small amount of partial cell attachment visible generally in about 2 h. It took about 10 days for cells to be completely attached. Cell growth accelerated incredibly, which could be passaged in about 2-4 days. Overall, post third-generation cells were good in terms of both morphology and proliferation. However, with the increase of passage number, cell growth slowed down in the plateau phase after the proliferation phase; cell growth and proliferation weakened gradually; and cell morphology also changed accordingly; showing markedly enlarged cell volume, which indicated slow cell entry into the involution phase (Figures 1a, b, c, d).

Flow Cytometry

The cells we extracted and cultured were not necessarily completely the purest BMSCs, which contained hematopoietic stem cells and non-hematopoietic stem cells. Premature initial medium replacement may result in some loss of adherent cells. Extension of medium replacement time allowed more solid and stable BMSCs adherence, but it may increase the adherent growth of some non-BMSCs. Generally, we replaced the medium initially at 48 h. Some suspended erythrocytes and cell debris could be found. Over time, some non-stromal stem cells ruptured to death, thus allowing extraction of purer BMSCs. We used flow cytometry to analyze three antigens and identify the cell surface expressions of specific antigens, of which CD44 and CD90 expressions were positive, and CD34 was negative (Figure 2). These indicated that the majority of cells we extracted and cultured were BMSCs.

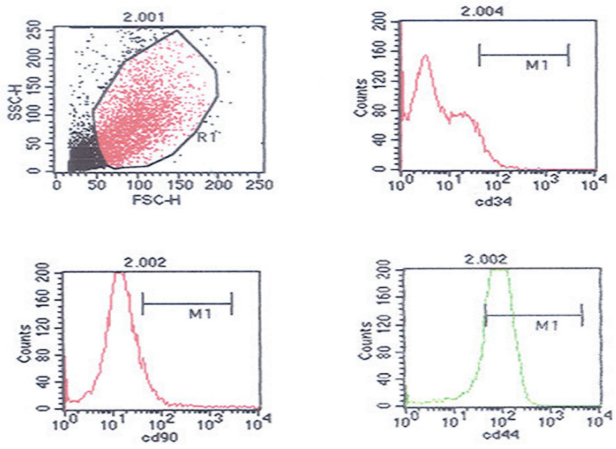


Figure 2: Cell surface marker CD44 D90 positive, CD34 negative expression via flow cytometry.

MTT Cell Proliferation Assay

The 96-well plate layered in advance was removed from the incubator, added with prepared MTT into 12 wells at 20 ul per well and placed in the incubator for 4 hours. Then, the tantalum material was removed, and 200 ul of DMSO was added to each well, followed by measurement of optical density at 490 nm (Figure 3).

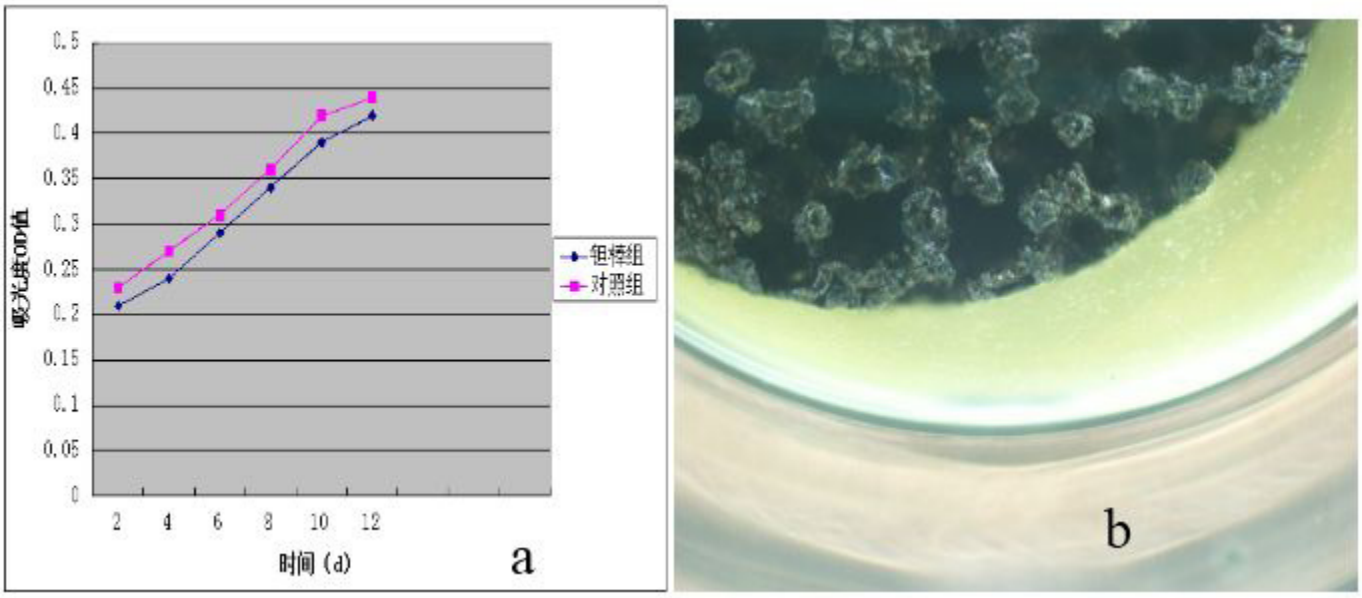


Figure 3: (a) The tantalum material group and the blank group had no significant difference in cell growth curve, may be related to the early pancreatic enzyme digestion cells for a long time cause the decrease in the number of cells, **(b)** We observed the number of the cells attached to the surface of tantalum material increased significantly by inverted microscope. The results showed that tantalum material has good biocompatibility, it had no toxicity to the growth of cell proliferation, and cells could be a good surface contact with the tantalum material and grew well.

SEM Observation of Material and Cell Co-Culture Results

Observation found that with the prolongation of porous tantalum and BMSC co-culture time, cells attached to the surface of porous tantalum differed completely in morphology and number. 5 d after co-culturing of canine BMSCs with porous tantalum material (a), no cell growth was noted around or on the surface of porous tantalum, and cell morphology did not change significantly. 10 d after co-culturing of canine BMSCs with porous tantalum (b), cells were in multi-protruding spindle or polygonal shape, at which cell junction was not inseparable. 15 d after co-culturing (c), with the increase of culture days, cells exhibited extremely active proliferation, which were arranged tightly to each other. Cells were interconnected and crawled into the pores of porous tantalum. Cells completely covered the surface of porous tantalum, and bulky overlapping was formed between cells. Clearly, good biological histocompatibility is one of the prominent advantages of porous tantalum (Figure 4).

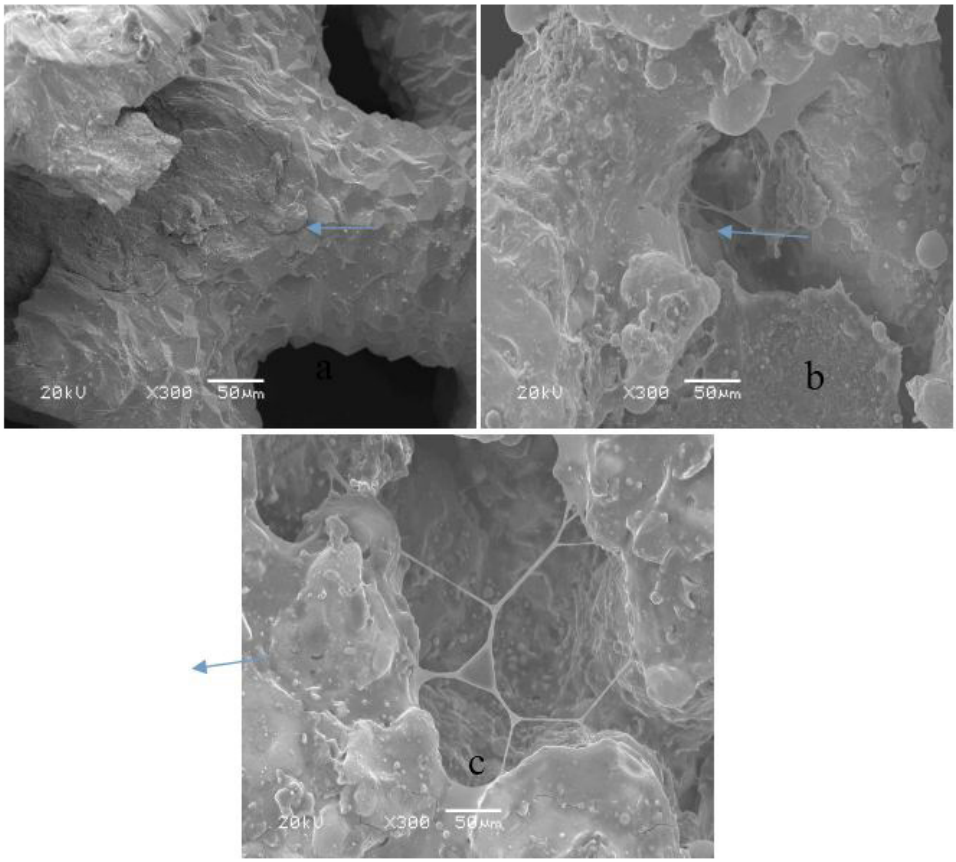


Figure 4: Scanning electron microscopy (× 300) tantalum co-cultured with cells **(a)** The BMSCs of dog co-cultured with tantalum for 5d, the surface of porous tantalum has not been seen cell growth, **(b)** The BMSCs of dog co-cultured with porous tantalum for 10d , the cells showed a plurality of projections spindle or polygonal, the connection between the cells is not close but looser, **(c)** The BMSCs of dog co-cultured with porous tantalum for 15d, the surface of porous tantalum presents exuberant cell proliferation , the cells coverage on the surface of porous tantalum, cell-cell connect into pieces.

In Vivo Compatibility Observation

The *in vitro* study proved that the porous tantalum had no rejection or toxicity against BMSCs and possessed good compatibility. We cut the porous tantalum into 0.6 cm long cylinders with a diameter of 0.4 cm and implanted them into the bone defect sites. 6 weeks after the implantation, the material presented clear boundary with bone tissue peripheries. In the pores of the porous tantalum, a small amount of bone tissue crawling was observed, as well as slight trabecular bone ingrowth. In the non-attached parts, voids and cracks surrounding pores were visible, while no tissue damage or rejection was noted around the material. 12 weeks after tantalum implantation, favorable growth of material with bone tissues was clearly observable over time. Substantial bone tissue ingrowth was noted on the surface and in the pores of the porous tantalum. Material pores were tightly connected to tissues; a large amount of trabecular bones in grew; and the porous tantalum fused into one with bone tissues (Figure 5).

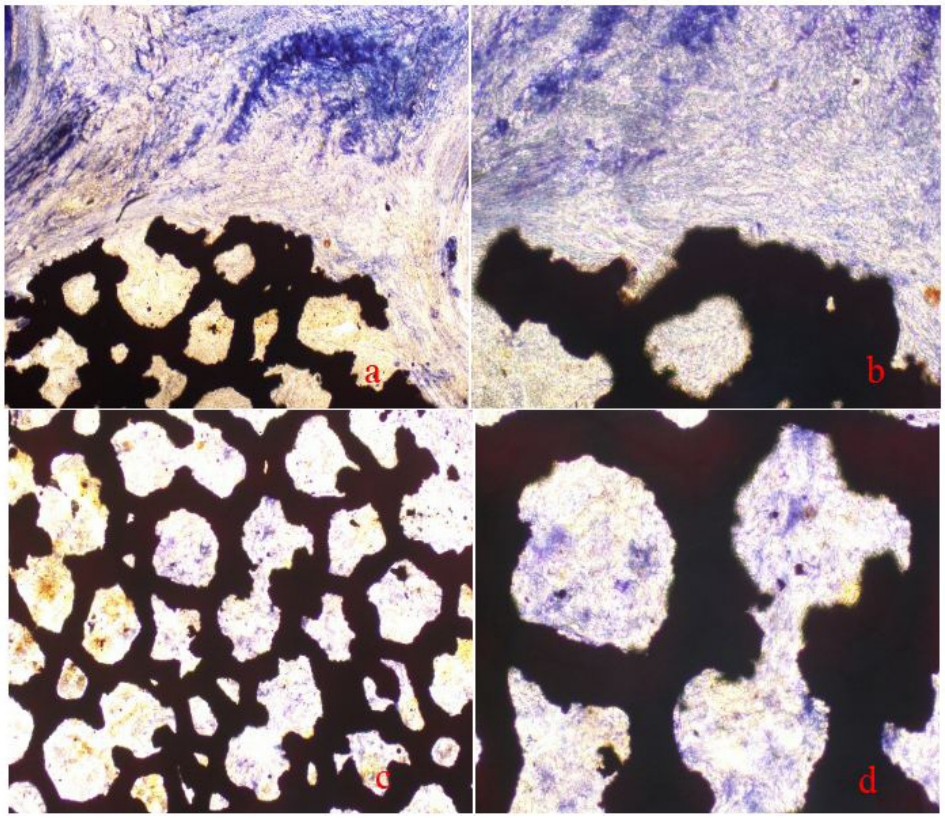


Figure 5: Porous tantalum implanted into dogs between 6 to12 weeks observe the morphology under microscope (× 40), (× 100) **(a)**The bound of the material and bone tissue was clear, **(b)** Less bone tissue crawled in the porous tantalum pores , and less trabecular bone ingrowth , void and cracks could be seen around the unattached part, **(c)** Porous Tantalum implanted into dogs for 12 weeks, with the extension of the time period , the bone tissue could be clearly observed that grow in the Materia well, **(d)** More bone tissue growth in the surface of porous tantalum and pores, material porosity connected with bone tissue closely , lots of bone trabecular grown in , bone tissue and porous tantalum grown into one

Discussion

Bone defects are partial or extensive bone damage caused by a variety of factors, which affect the body's physiological functions and eventually lead to deformity and disability. Over the years, treatment of bone defects has been troubled the clinical surgeons. Moreover, as the therapeutic efficacy is not desirable for every patient, they bear enormous psychological and economic burdens. With the rapid rise of medical technology, medical biomaterials, as a foreign implant, have gained the attention of most medical and biomaterial researchers. To clinically apply the biomaterials into certain parts of the body, they should first have good biocompatibility, cellular affinity and structural settings, so that they do not produce rejection and tissues can be grown adherently on their surface. Thus, biocompatibility and cellular affinity are the primary problems to be solved for all grafts.

In recent years, with in-depth study of BMSCs, many breakthroughs have been achieved in their application for treating various diseases such as bone or cartilage injury, heart disease, central nervous system injury, liver injury and spinal cord injury [4-11]. BMSCs have become one of the hottest topics in clinical research, and great achievements have been made on BMSCs in animal experiments and clinical studies. Over the years, BMSCs have been a major target in the research of bone tissue engineering. BMSCs have excellent multi-directional differentiation potential and proliferative capacity, which, most importantly, have the following advantages [12]: extensive source, simple extraction, minor trauma to the animal body and most importantly, simple cultivation of cells which are easily accessible in a large number. Ultimately, all these are proliferative capacity unique to cells. Featuring a wide range of differentiation, cells are extracted from animal body, cultured *In vitro* and induced for differentiation under special conditions. BMSCs can differentiate in multiple directions into mesenchymal cells such as chondrocytes, osteoblasts, cardiomyocytes, adipocytes and neurocytes; have strong self-renewal capacity and strong adhesion; and maintain the multi-directional differentiation potential after a long period of cultivation. According to experimental research, neither autologous nor allogeneic mesenchymal cells produce immunological rejection, indicating that stem cells can be used in clinical treatment of certain diseases, and orthopedic diseases are undoubtedly no exception. Therefore, BMSCs have broad research prospects.

In vitro adherent cultivation of BMSCs with porous tantalum rod material for detecting biocompatibility of porous tantalum rod is a simple and easy approach, which allows direction observation of results and repeatable operation. The biggest advantage is that it avoids the mutual interference of multiple complex uncontrollable factors when detected by *in vivo* implantation. Observation of biological reaction between cells and porous tantalum rod and their comparison with the chemotactic, proliferative and differentiation characteristics of cells from a mere cell and tantalum rod cultivation level allow the assessment of the influence of the material on cellular metabolism and cytotoxicity and the confirmation of compatibility of biomaterial in a relatively short time. Review of the literature found that such a method of examining material biocompatibility is a common approach [13-15].

Tantalum has been studied long time ago. With high biocompatibility, tantalum rod provides a stable support structure after implantation, and can prevent collapse of the femoral head while providing favorable conditions for the bone tissue restoration [16]. Mrosek, et al. [17] proved through rabbit experiment that porous tantalum combined with periosteum play a good role in restoring the cartilage defects. Tantalum has a favorable histocompatibility with human tissues, which does not produce irritation and rejection side effects and thus be widely used in medical research. Findlay et al.'s [18] experiment demonstrated that cells and pure tantalum have a good long-term compatibility, so tantalum has been applied in the field of clinical orthopedics. Rise of tantalum implants began with Tsao, et al.'s [19] report of a multicenter trial covering 98 cases (113 hips) treated with tantalum rod in 2005, which clearly stated that application of porous tantalum rod in ARCO stage II patients yielded good efficacy. Exploiting the high porosity (75-80%) of tantalum, which was 430 μm on average, the material was placed in the predefined specific site of animals for observation. For canine cylindrical porous tantalum implants, the pore bone in growth rates were 42%, 63% and 80% after 4 weeks, 16 weeks and 1 year from surgery, respectively, showing good fixation between the implant and the bone, which was suitable for fast human tissue ingrowth [20]. At present, the good biological characteristics of tantalum rod can provide effective structural support to bone defect sites; promote repair and reconstruction of bones at these sites; and delay and prevent further enlargement of defect range. Thus, tantalum rod is widely used in the treatment of certain human diseases. In this study, adherent growth of cells on the surface of porous tantalum material was observed by inverted microscope and electron microscope using *in vitro* BMSCs combined with independently developed tantalum material. Biocompatibility of porous tantalum with *in vivo* tissues and tissue proliferation were observed through hard tissue sections by *in vivo* femoral modeling and implantation of porous tantalum material at two-time points to illustrate the good biocompatibility between the two. Porous tantalum can be applied as a favorable medical bioscaffold material, which grows well with tissues *in vivo* and possesses good histocompatibility.

Conclusion

Cells we extracted are finalized indeed to be BMSCs. MTT assay clarifies the non-cytotoxicity of the porous tantalum material. Electron microscopy demonstrates that cells are attached tightly to the porous tantalum material. *In vivo* experiment indicates that the porous tantalum material has good histocompatibility, as well as bridging and proliferative effects. Porous tantalum material can promote the bone repair and growth after femoral head defects. Compared to BMSCs, the porous tantalum material exhibits better biocompatibility both *In vitro* and *in vivo*.

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